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(54) Title: RECOMBINANT PHAGES CAPABLE OF ENTERING HOST CELLS VIA SPECIFIC INTERACTION WITH AN ARTIFICIAL RECEPTOR

(57) Abstract: The invention relates to a genetically modified bacteriophage, pseudovirion or phagemid capable of entering a host cell by binding of its artificial ligand to an artificial receptor present on said host cell. The invention relates also to the use of the genetically modified bacteriophage, pseudovirion or phagemid and of the host cell to screen sequence libraries, including antibody library.

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Recombinant phages capable of entering host cells via specific interaction with an artificial receptor

The present invention relates to a recombinant bacteriophage, pseudovirion or phagemid that is capable of entering bacteria by specific binding to an artificial receptor. Said receptor does not comprise at its active binding site elements such as proteins or peptides that are derived from the natural receptor used in the specific initial bacteriophage - bacterium interaction.

Background of the invention

Bacteriophages, like bacteria, are very common in all natural environments. Bacteriophages (phages) are intracellular parasites. Bacteria and their phages have a common evolutionary history and phages may have adapted to their host species by multiple mechanisms. The phage genome may consist of double-stranded DNA, single-stranded DNA, double-stranded RNA or single-stranded RNA. Bacteriophages exist in several morphologies and can be spherical, cubic, filamentous, pleomorphic or tailed. Based on their life cycle, bacteriophages can be divided into three groups: the virulent phages capable of only lytic propagation (called lytic phages), the so-called temperate phages capable of either lytic propagation or lysogenic phase and the non-lysing phages where the mature phage is continuously extruded. The virulent life cycle of wild type phages consists of infection of the host cell, i.e. attachment to a specific receptor in the bacterial cell wall, followed by entering of the phage genome in the cell, replication of the phage genome, production of the phage structural components, phage assembly and release of the progeny phages after lysis of the host cell. In the lysogenic life cycle, the phage genome exists as a prophage resulting in coexistence of phage and host cell without lysis. Usually, this is achieved by integration of the phage genome into the bacterial chromosome. The life cycle of the non-lysing phages, like e.g. Bacteriophage M13, is similar to that of the lytic phages, but the infection is not followed by lysis.

Bacteriophages have been extensively used in biotechnology.

Phage genes or complete phages may be used to obtain lysis and/or killing of bacteria.

US4637980 describes the use of an *E. coli* strain containing defective temperature sensitive lambda lysogens as a method for cell disruption. Smith and coworkers (Smith *et al.*, 1987, *J. Gen. Microbiol.* 133: 1111-1126) describe the use of bacteriophages to treat diarrhoea in calves, caused by seven different bovine enteropathogenic strains of *E. coli.* WO95/27043 describes a method to treat infectious diseases caused by several bacterial genera, such as *Mycobacterium, Staphylococcus, Vibrio, Enterobacter, Enterococcus, Escherichia, Haemophilus, Neisseria, Pseudomonas, Shigella, Serratia, Salmonella* and *Streptococcus,* comprising the administration of bacteriophages with delayed inactivation by the animal host defence system. WO 98/51318 describes a diagnostic kit and a pharmaceutical composition, comprising bacteriophages to diagnose and tro treat bacterial diseases caused by bacteria, such as *Listeria, Klebsiella, Pneumococcus,, Moraxella,, Legionella, Edwardsiella, Yersinia, Proteus, Heliobacter, Salmonella, Chlamydia, Aeromonas and Renibacterium.*

Another application of bacteriophages is the *in vitro* selection of proteins displayed on the tip of filamentous phages on immobilised target (= biopanning), which is a powerful technique for the isolation of interacting protein-ligand pairs from large libraries, such as antibody libraries (for a recent review: Rodi and Makowski, 1999, *Curr. Opin. Biotechn.*, 10: 87-93). However, for optimal *in vitro* biopanning, a purified target protein is needed. Moreover, high quality of the library is a prerequisite for success. Enrichment for selfligated vector, phages carrying incomplete sequences, incorrect reading frames, deletions and amber stop codons are very often observed (Beekwilder et al, 1999, *Gene,* 228, 23-31 and de Bruin et al, 1999, *Nature Biotechnology,* 17: 397-399). In the search to avoid the problems encountered with panning using imperfect libraries, several alternative techniques, both bacteriophage based and non bacteriophage based,have been

developed. Non bacteriophage based techniques are, amongst others ribosome display (Dall'Acqua and Carter, 1998, *Curr. Opin. Struct. Biol.*, 8: 443-450) and the yeast two-hybrid system (Drees, 1999, *Curr. Opin. Chem. Biol.*, 3: 64-70). Bacteriophage based techniques comprise display on phage lambda, SIP (Spada and Pluckthun, 1997, *Biol. Chem.*, 378: 445-456; EP0614989) and CLAP (Malmborg et al, 1997, *J. Mol. Biol.*, 273: 544-551; WO9710330). SIP and CLAP are *in vivo* selection techniques and have the advantage that the F^+ *E.coli* host cells can only be infected by bacteriophages or pseudovirions when a matched pair is formed. Both systems are based on the fact that pilin on the F-pili of *E.coli* cells serve as the natural receptor for binding of the D2-domain of pIII from the phage (Deng *et al.*, 1999, *Virology*, 253:271-277). This results in retraction of the pilus, so that an interaction between the D1 domain of pIII with the TOL protein complex located in the *E.coli* cell membrane leads to the infection (Deng et al, 1999, *Virology*, 253: 271-277). SIP has the disadvantage that it only works for high affinities of the binding pairs and that each target needs to be cloned, expressed and purified as a fusion with the D2 domain of pIII. Therefore, with SIP, normally only one target can be screened at the time. For CLAP only small peptides (15-18 amino acids) can be expressed on the F-pilus, hence, this technique can only be used for small linear epitopes. An additional disadvantage is the need for modified M13 to avoid natural infection of host cells. Therefore, removal of the D2 domain of pIII is essential. This results in a truncated form of M13 and concomitant difficulties to obtain good titres.

It is known that bacteriophages use specific receptors on the host cell wall as a way to recognise the host cell and to start the infection process. In all the applications cited above, the propagation of phages, pseudovirions or phagemids is dependent on the use of the natural phage receptor, or part of it, on the host cell wall. For M13, mainly used in these systems, the natural receptor is pilin (Malmborg *et al.*, 1997, *J. Mol. Biol.* 273: 544-551). Other examples of natural receptors are lamB for bacteriophage lambda (Werts *et al,* 1994, *J. Bacteriol.* 176:

941-947), the outer membrane protein OmpA for bacteriophages K3, Ox2 and M1 (Montag *et al,* 1987, *J. Mol. Biol.,* 196: 165-174), the outer membrane proteins OmpF and Ttr for bacteriophage T2 (Montag *et al,* 1987, *J. Mol. Biol.,* 196, 165-174), the outer membrane protein OmpC for the T4 phage family (T4, Tula, Tulb) (Montag *et al.*,1990, *J. Mol. Biol.,* 216: 327-334). The T4 bacteriophage family is using a C-terminal region of protein 37 as natural ligand (Montag *et al.*, 1990, *J. Mol. Biol.,* 216: 327-334), bacteriophages T2, K3, Ox2 and M1 are using protein 38 as natural ligand (Montag *et al,* 1987, *J. Mol. Biol.,* 196, 165-174) whereas phage lambda is using the C-terminal portion of the lambda tail fibre protein as natural ligand (Wang *et al.*, 1998, *Res. Microbiol,* 149: 611-624). Bacteriophage - receptor independent phage binding to mammalian cells expressing the growth factor receptor ErbB2 followed by receptor mediated endocytosis was also described: Marks and collaborators (Poul and Marks, 1999, *J. Mol. Biol.*, 288: 203-211 and Becceril and Marks, 1999, *Biochem. Biophys. Res. Commun.*, 255: 386-393) successfully isolated phage capable of binding mammalian cells expressing the growth factor receptor ErbB2 and undergoing receptor mediated endocytosis by selection of a phage antibody library on breast tumour cells and recovery of infectious phage from within the cell. However, the phage could not propagate in the mammalian cell, and the detection of the cells carrying bacteriophage could only be realised in an indirect way, by expression green fluorescent protein as a reporter gene.

Summary of the invention

One aspect of the invention is a genetically modified bacteriophage, pseudovirion or phagemid that is not dependent upon its natural receptor or parts thereof for entering a host cell.

Another aspect of the invention is a genetically modified bacteriophage, pseudovirion or phagemid capable of entering a host cell by specific binding to an artificial receptor. These artificial receptors can be endogenous host cell proteins located at the bacterial surface, or parts thereof, that are normally not involved in

the bacteriophage - bacterium interaction, but it may also be heterologous proteins, preferentially fusion proteins displaying an oligo- or polypeptide on the bacterial surface. The genetically modified bacteriophage, pseudovirion or phagemid binds to the artificial receptor preferentially by an artificial ligand. A specific embodiment is a genetically modified bacteriophage that is not dependent upon OmpA, OmpC, OmpF, Ttr or pilin for interaction with and/or entering *E.coli.* A further specific embodiment is a genetically modified M13 bacteriophage, pseudovirion or phagemid that does not depend upon pilin, or fragments thereof for specific interaction with and/or entering of *E. coli.* Said M13 bacteriophage, pseudovirion or phagemid can enter both F^+ and F^- *E. coli* cells, dependent upon an artificial receptor that is displayed on the surface of said cells.

Still another aspect of the invention is a bacteriophage, pseudovirion or phagemid that enters the host cell mediated by an antigen - antibody reaction, whereby in the binding complex no proteins or parts of the natural receptor are involved.

A preferred embodiment of the invention is a genetically modified M13 phage, pseudovirion or phagemid displaying an antibody, preferentially the variable part of a camel heavy chain antibody for instance disclosed in international patent application WO 94/04678 and in Hamers-Casterman C et al Nature,vol 363, 3 June 1993.p 446-448, on its tip, which can enter an *E.coli* host cell, displaying the antigen, preferentially as an pOprI fusion protein or as a TolA fusion protein. The use of OprI as a protein for the expression of an amino acid sequence at the surface of the cell wall of a host cell is disclosed for example in international patent application WO 95/04079 which is incorporated herewith by reference. TolA has been described by Levensgood and Webster (J. Bacteriol., 171, 6600-6609, 1989).

A further aspect of the invention is the use of said bacteriophage, pseudovirion or phagemid for selective entering of a subpopulation of bacteria. Using the specific artificial receptor interaction, in a mixed culture, the bacteriophage, pseudovirion or phagemid will only enter those bacteria that carry said artificial receptor. By this,

the subpopulation of bacteria can be identified and/or eliminated. One embodiment of the invention is the specific elimination of pathogenic bacteria by directing a recombinant bacteriophage, pseudovirion or phagemid to a specific bacterial surface protein of said pathogenic bacteria. The pathogenic bacteria can be gram positive, gram negative or gram variable and can belong, amongst other to the genera *Aeromonas, Chlamydia Edwardsiella, Enterobacter, Enterococcus, Escherichia, Haemophilus, Heliobacter, Klebsiella, Legionella, Listeria, Moraxella,, Mycobacterium, Neisseria, Pneumococcus, Proteus, Pseudomonas, Renibacterium, Salmonella, Serratia, Shigella, Staphylococcus, Vibrio* or *Yersinia*, without that this summation is limitative.

Elimination can be obtained by the lytic cycle of the bacteriophage, but is not limited this method. Other methods of eliminating the host cell may be the production of a toxic product encoded by the recombinant bacteriophage genome in the host cell. A preferred embodiment is the production of barnase placed after an inducible promoter, such as the barnase - barstar cassette described by Jucovic and Hartley (*Protein engineering,* 8: 497-499, 1995).

Another aspect of the invention is a host cell, entered by the genetically modified bacteriophage, pseudovirion or phagemid. Such host cell comprises the nucleotide sequence encoding the artificial receptor and the nucleotide sequence encoding the artificial ligand. Such sequences may be expressed in the host cell in combination with marker sequences, especially sequences encoding antibiotic resistance genes. A preferred embodiment is an *E. coli* cell, preferentially transformed with a plasmid encoding a pOprI-fusion protein, more preferentially transformed with a plasmid derived from ptrc-OprI, carrying a genetically modified M13 phage, pseudovirion or phagemid, preferentially a pK7C3 derived phagemid, wherein said genetically modified M13 phage is modified, especially by *in vitro* construction, with a nucleotide sequence encoding a protein capable of specifically binding to the pOprI-fusion protein.

Another preferred embodiment is an *E. coli* cell, preferentially transformed with a

plasmid encoding a TolA-fusion protein, more preferentially transformed with a plasmid derived from pBAD-TOLA (figure 11), carrying a genetically modified M13 phage, pseudovirion or phagemid, preferentially a pK7C3 derived phagemid or a Fdtetdog derived phagemid, wherein said genetically modified M13 phage is modified, especially by *in vitro* construction, with a nucleotide sequence encoding a protein capable of specifically binding to the TolA-fusion protein.

In a particular embodiment of the invention, the OprI-fusion protein is carried out in introducing the nucleotide sequence of the fusion partner acting as the region for interaction with the ligand expressed on the bacteriophage, pseudovirion or phagemid, especially as disclosed in WO 95/04678.

Still a further aspect of the invention is the use of said bacteriophage, pseudovirion or phagemid to identify interacting proteins, including cases where none of the members of the interacting protein is known.

In different embodiments, the bacteriophage, pseudovirion or phagemid can be used to screen (1) a host cell, displaying a bait against a library of bacteriophages, pseudovirions or phagemids displaying the preys, (2) a bacteriophage, pseudovirion or phagemid displaying a bait against a library of host cells displaying the preys, (3) a library of bacteriophages, pseudovirions or phagemids displaying different preys or baits against a library of host cells, displaying different baits or preys (As illustrated in Figure 1).

A preferred embodiment is where pOprI is used as fusion partner for the display of bait or prey on the surface of the F$^-$ *E.coli* strains (Williams & Meynell 1971. Mol. Gen. Genet. 113: 222-227) such as DH5α and UT5600 as host cell and where the phagemid pK7C3 or phagemid Fdtetdog is used for cloning the prey or bait as a pIII fusion protein.

Another preferred embodiment is where TolA is used as fusion partner for the display of bait or prey on the surface of said *E. coli* strains and where the phagemid pK7C3 or the phagemid Fdtetdog is used for cloning the prey or bait as a pIII fusion protein. Said TolA-fusion may be at the carboxyterminal end of TolA,

possibly with a hinge region, or may result in a deletion of a smaller or lager part of TolA. Hinge region, as used here, is an amino acid sequence, which is not necessarily limited in length, and may be even a full-length protein such as OprI. One specific embodiment is a TolA-fusion in which the D1-binding domain of TolA has been deleted and replaced by the fusion partner that should be displayed; in this case, preferably a genetically modified M13 phage, pseudovirion or phagemid is used in which the D1 domain has been deleted. More preferably, in this case a Fdtetdog-D1 derived phagemid is used.

Another embodiment of the invention is the construction of a subtraction library, with the use of lytic bacteriophages, preferentially barnase expressing bacteriophages. In this embodiment, a part of the host cell library is recognised by lytic phages such as barnase expressing phages and killed upon recognition of the artificial receptor by the artificial ligand, entering of the bacteriophage, pseudovirion or phagemid and expression of the lytic gene.

Another aspect of the invention is a method for selecting artificial receptor - artificial ligand interactions, comprising:

- growing a host cell or a mixture of host cells displaying one or more artificial receptors,
- contacting said host cell or said mixture with a genetically modified bacteriophage, pseudovirion or phagemid or a mixture of genetically modified bacteriophages, pseudovirions or phagemids with one or more artificial ligands,
- selecting those cells that have been entered by one or more bacteriophages, pseudovirion of phagemid.

One embodiment of the invention is said method, whereby the selection is based on an antibiotic resistance marker. Another embodiment is said method whereby the cells are selected by killing of the host cell, preferentially by expression of barnase. A preferred embodiment is said method, whereby the host cell is an *E. coli* cell, displaying the artificial receptor as a pOprI fusion protein, and the

genetically modified bacteriophage, pseudovirion or phagemid is a genetically modified M13, displaying an artificial ligand as a pIII fusion protein.

Another preferred embodiment is said method, whereby the host cell is an *E. coli* cell, displaying the receptor as a TolA fusion protein, and the genetically modified bacteriophage, pseudovirion or phagemid is a genetically modified M13, displaying an artificial ligand as a pIII fusion protein. A specific embodiment is said method, whereby in said TolA-fusion the D1 bindings domain have been deleted and in said pIII fusion protein the D1 domain has been deleted.

Definitions

The following definitions are set forth to illustrate and define the meaning and scope of the various terms used to describe the invention herein.

Genetically modified bacteriophage: a bacteriophage of which the genome has been modified, at least by the introduction of the gene encoding for an artificial ligand. This introduction can be as a replacement of one of the endogenous genes or as an additional gene besides the endogenous genes.

Natural receptor: protein domain, protein or protein complex situated on the host cell wall, involved in the natural initial interaction between a bacteriophage and said host cell, whereby this interaction is followed by introduction of the genetic material of the bacteriophage into the host cell.

Artificial receptor: protein domain, protein, fusion protein or protein complex on the host cell wall ,whereby said protein domain, protein, fusion protein or protein complex does not contain one or more peptide fragments of at least 10 contiguous amino acids derived from the natural receptor of the bacteriophage used in the protein sequence or region that is involved in the interaction between bacteriophage, pseudovirion or phagemid and the artificial receptor.

Protein : encompasses peptide, protein, glycoprotein, lipoprotein or another form of modified protein, including chemically modified protein.

Protein complex: protein - protein complex, but also protein - compound complex, whereby said compound may be any chemical or biological compound, including simple or complex inorganic or organic molecules, peptido-mimetics, carbohydrates, nucleic acids or derivatives thereof.

Natural ligand: protein, protein domain or protein complex of the bacteriophage, pseudovirion, or phagemid involved in the natural initial interaction between said bacteriophage, pseudovirion, or phagemid, and a host cell, including recognition of and possibly binding to the natural receptor, whereby this interaction is followed by introduction of the genetic material of the bacteriophage into the host cell.

Artificial ligand: protein, protein domain or protein complex of the bacteriophage, pseudovirion, or phagemid, whereby said protein domain, protein, fusion protein or protein complex does not contain one or more peptide fragments of at least 10 contiguous amino acids derived from the natural ligand of the bacteriophage in the protein sequence or region that is involved in the interaction between bacteriophage, pseudovirion or phagemid and the artificial receptor.

Host cell: any bacterial cell that can allow a bacteriophage, pseudovirion or phagemid to enter said cell after interaction of a said bacteriophage, pseudovirion or phagemid with a natural or artificial receptor. As example, host cells include gram-negative or gram-positive bacteria, especially including E coli cells and in particular F^- cells which do not permit entering of bacteriophages, pseudovirions or phagemids through the pillin mechanism.

Entering bacteria: means that the bacteriophage, pseudovirion or phagemid can enter as a whole or as a part (e.g. only the genetic material) the host cell after specific binding to the artificial receptor. The mechanism by which the material is entering the host cell is not limited to specific ways and can be amongst others an active infection process or a passive uptake by the host cell. Methods for determination of the specific binding of the artificial ligand with the artificial receptor are illustrated in the examples.

Specific binding: means that the initial step of the entering is mediated by a specific interaction between the artificial receptor on the host cell wall and the artificial ligand of the bacteriophage, pseudovirion or phagemid. This specific interaction is preferentially a protein - protein interaction. This entering after specific interaction should be distinguished from the Calcium dependent pilus independent infection that can be detected with M13 bacteriophages in which the second N-terminal domain of gIIIp has been removed (Krebber *et al.*, 1997, *J. Mol. Biol.* 268: 607-618).

According to particular embodiments, the invention relates to a genetically modified host cell, transformed with a nucleotide sequence encoding an artificial receptor in conditions enabling that the artificial receptor be expressed at the surface of the host cell, said host cell being further transformed with a nucleotide sequence encoding said artificial ligand whereby said nucleotide sequence encoding the ligand entered the host cell as a consequence of the interaction between said artificial ligand and a protein sequence or region on said artificial receptor.

Particular genetically modified host cells are those wherein the nucleotide sequence encoding the artificial receptor and/or the nucleotide sequence encoding the artificial ligand are not initially known.

According to another specific embodiment, the genetically modified host cell is a gram-negative bacterium, especially an E coli cell of the F^- type. It should be noted, that, after binding of the bacteriophage to its artificial receptor, the F^- phenotype and/or genotype may be reconverted to a F^+ type. Such reconversion may be realized by, as a non-limiting example, temperature induction (Novotny and Lavin, J. Bacteriol. 107, 671-682, 1971), by induction of one or more genes encoding essential pilus proteins such as pilin, or by transfer of the pilus encoding genetic material by conjugation.

According to another particular embodiment, the genetically modified host cell is a transformed cell wherein the nucleotide sequences of the artificial receptor and

the nucleotide sequence for the artificial ligand are respectively coding sequences of an antibody or a functional fragment thereof and coding sequence of an antigen, or are respectively coding sequences of an antigen and coding sequence of antibody or a functional fragment thereof.

In said genetically modified host cell of the invention, the functional antibody fragment can be a variable fragment of an antibody, encompassing four-chain antibodies or two-chain antibody as defined in international patent application WO 94/04678, including native or modified, especially truncated chains thereof. In a preferred embodiment the variable chain is a VHH fragment of a camelid antibody or a functional portion of said VHH, as disclosed in the above cited patent application which is incorporated by reference.

The invention relates also to the above defined genetically modified host cell, wherein the nucleotide sequence encoding the artificial receptor comprises a sequence encoding OprI or TolA or a part of OprI or TolA sufficient to enable the exposure, at the surface of the host cell, of a protein sequence or region capable of interacting with the artificial ligand.

A further object of the invention is a kit comprising a genetically modified host cell according to the above proposed definitions and specific embodiments or comprising a host cell and/or a bacteriophage, pseudovirion or phagemid and/or means including a cloning vector enabling the construction of said host cell and/or a bacteriophage, pseudovirion or phagemid according to the above definitions.

A particular kit is designed to be used for in vivo panning of antibody or antibody fragment library, or antigenic sequences library.

Said kit can also be used for the simultaneous in vivo panning of both an antibody fragment library, and an antigenic sequences library.

The invention therefore provides means for the identification of target sequences or molecules including especially amino acid sequences capable of interacting with a determined receptor, whether the nature or sequences of said receptor is

known or unknown. Especially the invention can be used for the identification of therapeutic targets.

Short description of the Figures

Figure 1 gives a schematic representation of the screening of a proteome expression library against a camel VHH anti-proteome antibody library.

Figure 2: schematic representation of phagemid pK7C3.

Figure 3: schematic representation of plasmid ptrc-OprI

Figure 4: Results of the Western blot. From left to right: lane 1 shows the molecular weight markers. Lane 2 and 3 show the total lysate of *E. coli,* transformed with ptrc-OprI, respectively after growth in LB (lane 2) and M9 (lane 3). Lane 4 and 5 show the total lysate of *E. coli,* transformed with ptrc-OprI- OVA, respectively after growth in LB (lane 4) and M9 (lane 5). Proteins are visualised with anti-OprI, as described in example II.

Figure 5 (a and b): Schematic representation of barnase activation by inversion of the expression cassette, due to integrase activity induced by heat shock.

Figure 6: sequence of binders for ovalbumin obtained by biopanning and PICUP

Figure 7: schematic representation of the phage vector Fdtetdog

Figure 8: schematic representation of the phage vector Fdtetdog were the D1 domain in pIII is deleted

Figure 9: schematic representation of the phage vector Fdtetdog were the D2 domain in pIII is deleted

Figure 10: schematic representation of the plasmid pBAD-OprI

Figure 11: schematic representation of the plasmid pBAD-TOLA.

Examples

Example I: Construction of M13 pseudovirions displaying Camel Heavy Chain antibodies (VHH)

Immunisation of camels

A camel (B) was immunised with 1 mg hen-egg ovalbumin (Sigma) in the

presence of complete Freund adjuvant, and boosted in the presence of incomplete Freund adjuvant at days 7,14, 28, 35 and 42.

Anticoagulated blood was collected for lymphocyte isolation on day 45. This results in VHH library CAMELB

Construction of phagemid library

Peripheral blood lymphocytes were prepared using Unisep (WAK Chemie, Germany). The camelid heavy chain antibodies (VHH's) from 10^7 lymphocytes were cloned after RT-PCR amplification in the NcoI-NotI site of the pK7C3 vector (Figure 2) and transformed in TG1 (Lauwereys *et al*, 1998, *the EMBO Journal*, 17 : 3512-3520). The primers for the amplification are CATGCGATGACTCGCGGCCCAGCCGGCCATGGC and GTGTGCGGCCGCTGAGGAGACRGTGACCWG.

The pK7C3 vector is a pHEN4 (Ghahroudi *et al*, 1997, *FEBS letters*, 414: 521-526) derivative where the ampicillin resistance gene was replaced by the chloramphenicol resistance gene and the haemaglutinin tag was replaced by a histidine and c-myc tag (Ghahroudi et al, 1997, *FEBS letters*, 414: 521-526)

Construction of M13 pseudovirions displaying camel VHH

VHH's from the CAMELB library were expressed on phage after infection of the library with M13KO7 helper phage (pK7C3-VHHB) as described by Ghahroudi et al, 1997, *FEBS letters*, 414: 521-526. A library of 3 x 10^6 individual colonies was obtained of which 85% had the correct insert size, and 90% of these could produce a fusion protein between VHH and pIII.

Selection of ovalbumin specific pseudovirions by biopanning

The CAMELB library was panned for the presence of binders on ovalbumin coated in wells of microtitre plates (10µg ovalbumin / well). Bound phages were eluted and allowed to infect TG1 cells (Stratagene). After two or three rounds of panning, individual colonies were grown, periplasmic extracts were prepared and screened for the presence of ovalbumin binders in ELISA. (Skerra and Pluckthun, 1988, *Science*, 240: 1038-1041). The plasmid of these binders was prepared and

sequenced. We obtained 2 VHH binders of which 1DBOVA1 (DVQLVESGGGSVPAGSLRLSCAVSGYTYENRYMAWFRQAPGKEREGVAAIWR GGNNPYYADSVKGRFTISQDNAKNIVSLLMNSLKPEDTAIYYCAAQAGRFSGPLY ESTYDYWGQGTQVTVSS) was the most abundant.

Titre determination

The titre of the phages was determined by incubation of 150 µl TG1 (F^+) cells at OD600nm= 0.5 with 10 µl of phages of different dilutions, for 30 minutes at 37°C. This was plated on LB-agar plates containing 25 µg/ml chloramphenicol and 2% glucose.

The background for infection of DH5α (Gibco BRL) was determined under the same conditions as described above for TG1.

Preparation of the phages

Cultures of TG1 containing pK7C3-VHHB or 1DBOVA1 were grown at 37°C in 100 ml 2xTY medium containing 2% glucose, and 25 µg/ml chloramphenicol, until the OD600nm reached 0.5. M13KO7 phages (10^{12}) were added and the mixture was incubated in a water bath at 37°C for 2 x 30 minutes, first without shaking, then with shaking at 100 rpm. The culture was centrifuged (15', 4300 rpm, room temperature). The bacterial pellet was dissolved in 600 ml of 2xTY medium containing 25 µg/ml chloramphenicol and 25 µg/ml kanamycin, and incubated overnight at 30°C, vigorously shaking at 250 rpm.

These overnight cultures were centrifuged for 15 minutes at 4300 rpm at 4°C. Phages were precipitated for 1 hour on ice with PEG (20% poly-ethylene-glycol and 1.5 M NaCl), pelleted by centrifugation (30', 4300 rpm, 4°C), dissolved in 10 ml PBS and centrifuged for another 10 minutes at 4300 rpm and 4°C. The supernatant was loaded on 2 ml Ni-NTA (QIAGEN), washed extensively with 50 mM Na_2HPO_4, 1M NaCl pH= 7.0, eluted with 50 mM NaAc, 1M NaCl pH= 4.5 and neutralised with 1 M Tris pH= 7.4. Phages were again PEG precipitated by immediate centrifugation for 30 minutes at 4300 rpm and 4°C after PEG addition. The pellet (invisible) was dissolved in 1 ml PBS + 100 µl PBS-caseine. 15%

glycerol was added and the phages were stored at –80°C for maximally 1 week, until further use.

Example II: display of OprI and OprI-ovalbumin fusion protein on *E. coli*

The ptrc-OprI (Cote-Sierra *et al.*, 1998, *Gene*, 221:25-34; Figure 3) or ptrc-OprI-ova (obtained by amplifying the gene encoding for hen-egg ovalbumin, digesting the product with BamHI and EcoRI and cloning the digest in BglII/EcoRI digested ptrc-OprI) plasmids were transformed in E.coli Top10F' (Invitrogen) and tested for expression in M9CAA and LB-medium in Western blot. Cells were induced with 1 mM ITPG (Calbiochem) at OD600nm = 0.6 and grown overnight at 37°C on a rotary shaker at 200 rpm. Cells were centrifuged and concentrated 10-fold. Total cell lysates, obtained by sonication were loaded on a 12% SDS-PAGE and transferred to nitrocellulose for Western blotting. Transferred proteins were detected using a monoclonal anti-OprI antibody QB2 (De Vos D. et al, Journal of general microbiology 1993, 139: 2215-2223). An anti-mouse IgG conjugated with alkaline phosphatase (Sigma) was applied and the blots were developed with the BCIP/NBT substrate. The results are shown in Figure 4. A band at the position of intact fusion protein is clearly observed. However, large amounts of degradation products demonstrate the instability of the pOprI-ova form. Since these degradation products might interfere with the infection, conditions for growth and infection were optimised, amongst others by the use of UT5600 (F^-, *ara-14, leuB6, azi-6, lacY1, proC14, tsx-67, entA403, trpE38, rfbD1, rpsL109, xyl-5, mtl-1, thi1, Δ ompT, fepC266*) (Biolabs). UT5600 is an outer membrane protease T-deficient *E. coli* strain, which was used for the stable presentation of Ig scFv fusions (Maurer and Meyer, *J. Bacteriol.*, 1997, 179: 794-804)

Example III: receptor independent entering of *E.coli* by M13 pseudovirions.

Cultures of *E.coli* strain DH5α containing ptrc-OprI (Cote-Sierra et al, 1998, *Gene*, 221: 25-34) or ptrc-OprI-OVA (indicated as DH5α{ptrc-OprI-OVA}) were incubated

at 37°C at 220 rpm until the OD600nm reached 0.6. Cells were centrifuged at 4300 rpm for 5 minutes and resuspended in the original volume and in the same medium (= washed cells*). A fraction of the cells was induced with 1 mM IPTG and grown at 37°C for another 4 hours (**).

To test the pilus independent entering of *E.coli* displaying ovalbumin on its surface by M13 phages displaying ovalbumin specific antibodies, we incubated 150 µl of *E.coli* cells with 10 µl phages from pK7C3-VHHB of different dilutions for 1 hour at 37°C without shaking.

Infection of *E.coli* was screened for by selection of incubation mixtures on LB-agar plates containing 100 µg/ml ampicilline, 25 µg/ml chloramphenicol and 2% glucose.

Individual colonies were screened in ELISA. Therefore, large single colonies (resistant to ampicilline and chloramphenicol) were inoculated in 10 ml TB medium containing 0.1% glucose, 100 µg/ml ampicilline and 25 µg/ml chloramphenicol for 8 hours. IPTG was added at a final concentration of 10 mM and the cultures were grown overnight at 37°C at 200 rpm.

Individual colonies in TG1 and DH5α were picked and grown in 10 ml TB medium containing 0.1% glucose and 25 µg/ml chloramphenicol for 4 hours. IPTG was added at a final concentration of 1 mM and the cultures were grown overnight at 28°C at 200 rpm.

Periplasmic fractions were prepared by pelleting the overnight cultures, and dissolving the pellet in 200 µl TES (0.2 M Tris-HCl, pH= 8.0, 0.5 mM EDTA, 0.5 M sucrose). This was incubated on ice for 20 minutes. 300 µl TES/4 was added and incubated at 4°C for 25 minutes. This suspension was centrifuged for 25 minutes at maximal speed in an eppendorf centrifuge and the supernatant was used for testing in ELISA.

Periplasmic fractions were tested in NUNC-plates coated overnight with ovalbumin (5 µg/ml) or casein as a negative control (1% w/v in PBS) and blocked overnight with 1% (w/v) casein. Samples were incubated for 2 hours at room temperature

and ovalbumin binding VHH's were detected with a mouse anti-Histidine-tag (SEROTEC), anti-mouse-alkaline phosphatase conjugate (Sigma) and a chromogenic substrate (Sigma). The results are summarised in Table I

Table1	Positives in ELISA
TG1	1/337
DH5α	0/7
DH5α + ptrc-OprI	2/141
DH5α{ptrc-OprI-OVA}	7/16, 17/38, 7/24
DH5α{ptrc-OprI-OVA}, washed*	14/19
DH5α {ptrc-OprI-ova} + IPTG**	3/12

Table 1: The numbers indicate the number of positive clones in ELISA versus the number of clones that were tested. Extracts were scored positive if the OD405nm was at least double the OD of the background (coated caseine at 1%). Numbers separated by a comma are from independent experiments.

*: The cells were washed 1 time with fresh medium before infection with phages as described above.

**: Cells were induced with IPTG as described above.

Example IV: receptor independent entering of *E.coli* by pseudovirions is specific for the artificial receptor

150 µl of washed UT5600 containing ptrc-OprI-OVA (indicated as UT5600{ptrc-OprI-OVA}) or DH5α{ptrc-OprI-OVA} cells at OD600nm= 0.6 were incubated with 10 µl phages of pK7C3-VHHB of different dilutions for 1 hour at 37°C without shaking.

The same experiment was repeated after pre-incubation of the phages with 1ml ovalbumin (2mg/ml) for 1 hour at room temperature. The phages were mixed with 150 µl of washed UT5600{ptrc-OprI-OVA} or DH5α{ptrc-OprI-OVA} cells at OD600nm= 0.6 and incubated for 1 hour at 37°C.

This was plated on LB agar plates containing 25 µg/ml chloramphenicol, 100 µg/ml ampicilline and 2% glucose. Expression of 25-45 clones in UT5600 cells and DH5α was carried out as described above.

The results are summarised in Table 2.

Table2	**DH5α** c^r, a^r	**UT5600** c^r, a^r
E.coli **(ptrc-OprI-ova) + pK7C3-VHHB phages**	150	90, 73
E.coli **(ptrc-OprI-ova) + ovalbumin pretreated pK7C3-VHHB phages**	1	2, 2

Table 2: number of colonies on plates after infection of washed UT5600{ptrc-OprI-OVA} or DH5α{ptrc-OprI-OVA} cells with phages with or without pre-incubation with ovalbumin. Numbers separated by a comma are from independent experiments. c^r: chloramphenicol resistant; a^r: ampicilline resistant

Infection of washed UT5600{ptrc-OprI-OVA} or DH5α{ptrc-OprI-OVA} cells with phages from pK7C3-VHHB pre-incubated with hen-egg ovalbumin protein, reduced the number of transformants significantly, which means that infection is dependent upon ovalbumin display on the host cell wall.

Example V: Survival of *E.coli* cells and phages upon coincubation

150 µl of washed UT5600{ptrc-OprI-OVA} or DH5α{ptrc-OprI-OVA} cells at OD600nm= 0.6 were incubated with 10 µl phages of PK7C3-VHHB for 1 hour at 37°C without shaking.

This was plated on LB agar plates containing 25 µg/ml chloramphenicol, 100 µg/ml ampicilline and 2% glucose (C).

Cells were also checked for survival upon growth (A) and upon incubation with phages (B) by dilution and plating on LB agar plates containing 100 µg/ml ampicilline and 2% glucose. The results are shown in Table 3.

Table3 number of cells	DH5α	UT5600
Before incubation (A)	2×10^8	10^9
After incubation (B)	5×10^7	3×10^8
entered (C)	64	150

Table3: Number of cells upon incubation of washed UT5600{ptrc-OprI-OVA}_or DH5α{ptrc-OprI-OVA} cells with pK7C3-VHHB phages.

The titre of pK7C3-VHHB phages was determined before incubation with washed DH5α{ptrc-OprI-OVA} cells. Cells were centrifuged after incubation for 1 hour at 37°C and the supernatant was used to determine the titre of unentered phages. The titres were determined by incubation of 150 µl TG1 cells at OD600nm= 0.5 with 10 µl of phages of different dilutions for 30 minutes at 37°C. This was plated on LB-agar plates containing 25 µg/ml chloramphenicol and 2% glucose. The number of transformants are listed in Table4.

Table 4	number of phages
Before incubation	5.6×10^7
After incubation	1.2×10^7

Table4: Number of pK7C3-VHHB phages before and after incubation with washed DH5α{ptrc-OprI-OVA} cells

UT5600{ptrc-OprI-OVA} or DH5α{ptrc-OprI-OVA} cells survived very well when incubated with and entered by pK7C3-VHHB phages. The pK7C3-VHHB phages which did not enter UT5600{ptrc-OprI-OVA} or DH5α{ptrc-OprI-OVA} cells are still able to infect TG1 cells and are therefore stable under the conditions used.

Example VI: Individual *E.coli* cells displaying ovalbumin on the surface are entered by a single ovalbumin specific phage.

Positive clones were selected from experiment 2 for UT5600 (clone number 10, 11, 12, 13, 16, 17). They were inoculated in 5 ml LB containing 25 µg/ml chloramphenicol, and grown overnight at 37°C. Plasmid was prepared, transformed in TG1 and plated on LB agar plates containing 25 µg/ml chloramphenicol and 2% glucose. Individual colonies were tested in ELISA as described before (in TB containing 25 µg/ml chloramphenicol and 0.1% glucose):

Table5	Positives in ELISA
10	8/8
11	8/8
12	8/8
13	7/8
16	8/8
17	8/8

Table 5: number of positive clones in ELISA versus the number of clones that were tested for individual colonies.

Individual positive clones were selected and grown overnight for plasmid preparation. After transformation in TG1 individual colonies were tested in ELISA. All clones scored positive, therefore we can be sure that ovalbumin specific

phages have entered the cell. Colony PCR on these individual colonies showed that they have the same length if they originate from the same original clone.

Example VII: Optimisation of the conditions of receptor independent entering

Cells of DH5α{ptrc-OprI-OVA} and UT5600{ptrc-OprI-OVA} were grown at 37°C. A 150 µl sample was removed at different time intervals, washed and 5×10^8 phages of pK7C3-VHHB were added. This suspension was incubated for 1 hour at 37°C and plated on LB-agar plates with 2% glucose, 25 µg/ml chloramphenicol and 100 µg/ml ampicillin. Single colonies were tested in ELISA as described above. The results are shown in Table 6a and 6b.

UT5600, Table 6a

Time of growth (minutes)	**OD600 nm**	**tfu**	**Positives in ELISA pK7C3-VHHB**
30	0.062	20	7/15
60	0.076	56	7/15
90	0.142	100	**11/15, 2/3**
120	0.273	130	**11/16**
150	0.555	150	8/15
210	1.24	120	2/13
270	2.25	30	7/12

DH5α, Table 6b

Time of growth (minutes)	OD600 nm	tfu	Positives in ELISA pK7C3-VHHB
60	0.010	0	-
120	0.038	23	5/13
210	0.197	97	3/8
270	0.600	84	3/5
300	0.665	64	2/8

Tables 6a and 6b: Number of positive clones in ELISA versus the number of clones that were tested and the number of transformants (tfu) as a function of the OD600nm of the cells. Numbers separated by a comma are from independent experiments.

Cells of DH5α{ptrc-OprI-OVA} and UT5600{ptrc-OprI-OVA} were grown at 37°C. Increasing concentrations of pK7C3-VHHB phages were added to 150 µl of washed cells at OD600nm= 0.2-0.3 for UT5600{ptrc-OprI-OVA} and 0.6 for DH5α{ptrc-OprI-OVA}. This mixture was incubated for 1 hour at 37°C and plated on LB-agar plates with 2% glucose, 25 µg/ml chloramphenicol (Chl) and 100 µg/ml ampicillin (Amp). Single colonies were tested in ELISA as described before.

UT5600, Table7a

Number of phages added	Number of transformants on Amp/Chl	Number of positives in ELISA	Number of positives in ELISA (%)
7×10^6	30	0/2	-
1×10^7	20	0/2	-
7×10^7	85, 90, 150	0/5, 1/24, 2/24	-, 4, 8
1×10^8	70	¼	25
4×10^8	67	4/29	14
7×10^8	300, 300	18/24, 20/20	75, 100

DH5α, Table7b

Number of phages added	Number of transformants on Amp/Chl	Number of positives in ELISA	Number of positives in ELISA (%)
5×10^7	85	10/28	36
2×10^8	250	21/43	49
5×10^8	110	17/40	43

Table7a-b: Increasing concentrations of pK7C3-VHHB phages were mixed with 150 µl UT5600{ptrc-OprI-OVA} at OD600nm= 0.2-0.3 or with DH5α{ptrc-OprI-

OVA} cells at OD600nm= 0.6. Individual colonies were tested in ELISA. Numbers separated by a comma are from independent experiments.

Optimal conditions for uptake of phages were tested by mixing pK7C3-VHHB phages with UT5600{ptrc-OprI-OVA} or DH5α{ptrc-OprI-OVA} cells at different optical densities. Individual colonies were tested in ELISA. For UT5600{ptrc-OprI-OVA} the optimal density is between 0.15 and 0.3 and for DH5α{ptrc-OprI-OVA} between 0.2 and 0.6. UT5600{ptrc-OprI-OVA} cells grow much faster than DH5α{ptrc-OprI-OVA} and are easier infected by anti-ovalbumin expressing phages (positive clones in ELISA) (73% versus 30-50% table6a-b). When increasing amounts of phages were mixed with UT5600[ptrc-OprI-OVA] cells (OD600nm= 0.2-0.3), more positive clones were obtained in ELISA (table7a). 75-100% positive clones were obtained when 5-10 x 10^8 phages were mixed with 0.5 x 10^8 UT5600{ptrc-OprI-OVA} cells resulting in 150-500 individual colonies. However, in DH5α{ptrc-OprI-OVA}, no increase in positive clones in ELISA was observed upon addition of increasing amounts of phages (table7b).

Example VIII: selective elimination by killer phages

Barnase is a extracellular ribonuclease from *Bacillus amyloliquefaciens* (Hartley & Rogerson 1972, Prep. Biochem. 2: 229-242). A very low level of intracellular expression of barnase in *E.coli* is lethal because barnase depolymerizes the RNA of its host. Jucovic & Hartley developed a tightly controlled system (pMI47a) for the intracellular expression of barnase in *E.coli* (*Protein engineering*, 8: 497-499, 1995). The plasmid encodes barstar (a strong polypeptide inhibitor of barnase) under the transcriptional control of the Tac promotor. A barnase gene (without secretion signal) has been cloned in the inverse orientation downstream from barstar. In pMI47a, the Tac promotor is followed by attP, followed by barstar, followed by the inversed gene for barnase, followed by attB. AttP and attB are derived from the phage lambda attachment site. pMI47a is not toxic for *E.coli* because it overproduces barstar and no barnase (OFF configuration, figure 5a).

The Integrase protein (INT function) from phage lambda recognises the attB and attP sequences and inverses the DNA fragment that is located between the attB and attP sites *in vivo*. In the resulting plasmid pMI47b the Tac promotor is followed by attR, followed by barnase, followed by the inversed gene for barstar, followed by attL. Sites attR and attL are the products of recombination between attP and attB (Jucovic & Hartley, 1995, Protein Engineering 8: 497-499). This plasmid is toxic for *E.coli* because it produces active barnase in the cytoplasm of the host (ON configuration, figure 5b). The system can be switched in vivo from the OFF to the ON configuration in the *E.coli* strain D1210HP (supE44 ara14 galK2 lacY1 D(gpt-proA)62 rpsL20 (Str^r) xyl-5 mtl-1 recA13 D(mrcC-mrr)HsdS$^-$(r$^-$m$^-$) lacI^q LacY$^+$ lxis-kil-cI857) (Stratagene) by a short incubation at 42°C. This strain encodes the integrase function (Int; Ixis) from phage lambda, whereas D1210 doesn't have this function.

A conditionally lethal phage particle was made by cloning the conditionally lethal cassette of pMI47a into a phagemid. A DNA fragment of pMI47a including the Tac promotor, followed by attP, followed by barstar, followed by the inversed gene for barnase, and followed by attB was amplified by PCR. This PCR product was cloned as a blunt end fragment within the EcoRI site of the pK7C3-1DBOVA1 vector (example I) to give pK7C3BB-1DBOVA1 (Before ligation, pK7C3-1DBOVA1 was linearized with EcoRI and filled-in with Klenow DNA polymerase).

Plasmids of pK7C3BB-1DBOVA1, pK7C3, ptrc-OprI-OVA and pMI47a were transformed in D1210 and D1210HP electrocompetent cells. Individual colonies were grown in LB with 25 µg/ml chloramphenicol or 100 µg/ml ampicilline and 2% glucose at 37°C until the OD600nm= 1.2-1.3. Half of the culture was exposed to thermal induction (15 minutes at 42°C). Both fractions (before and after induction) were spread after appropriate dilutions, on LB agar plates containing 25 µg/ml chloramphenicol or 100 µg/ml ampicilline and 2% glucose. The numbers of transformants were counted and are listed in table 8.

construct	*E.coli* strain	temperature	Number of transformants/ml
PMI47a	D1210	37°C	3×10^8 Amp^r
		37°C-42°C	2×10^8 Amp^r
	D1210HP	37°C	2×10^8 Amp^r
		37°C-42°C	280 Amp^r
Ptrc-OprI-OVA	D1210	37°C	3×10^8 Amp^r
		37°C-42°C	2×10^8 Amp^r
	D1210HP	37°C	3×10^8 Amp^r
		37°C-42°C	2×10^8 Amp^r
pK7C3	D1210	37°C	5×10^8 Chl^r
		37°C-42°C	4×10^8 Chl^r
	D1210HP	37°C	6×10^8 Chl^r
		37°C-42°C	3×10^8 Chl^r
pK7C3BB-1DBOVA1	D1210	37°C	4×10^8 Chl^r
		37°C-42°C	3×10^8 Chl^r
	D1210HP	37°C	5×10^8 Chl^r
		37°C-42°C	560 Chl^r

Table 8: Number of transformants with or without thermoinduction of D1210 or D1210HP cells containing PMI47a, ptrc-OprI-OVA, pK7C3 or pK7C3BB-1DBOVA1.

The results show that D1210 cells (lacking the Int gene) transformed with PMI47a, ptrc-OprI-OVA, pK7C3 or pK7C3BB-1DBOVA1 survive well upon thermoinduction, which indicates that PMI47a, ptrc-OprI-OVA, pK7C3 and pK7C3BB-1DBOVA1 are

not harmful for *E.coli*. PMI47a, ptrc-OprI-OVA, pK7C3 and pK7C3BB-1DBOVA1 can be transformed and maintained in D1210HP if the cells are maintained at 37°C (OFF configuration). However, when cells are incubated at 42°C for 15 minutes (ON configuration), the integrase function is activated, and D1210HP cells containing PMI47a or pK7C3BB-1DBOVA1 do not longer survive. These experiments show that the phagemid pK7C3BB-1DBOVA1 is toxic for *E.coli* strain D1210HP if inversion is induced by thermoinduction of the Int gene.

Elimination upon recognition of an artificial receptor

D1210 and D1210HP electrocompetent cells were transformed with ptrc-OprI-OVA. A single colony was used to inoculate a culture in LB containing 100 µg/ml ampicilline. Phages of pK7C3-1DBOVA1 or pK7C3BB-1DBOVA1 were prepared as described above. 150 µl of washed cells were incubated with 5 x 10^8 pK7C3-1DBOVA1 or pK7C3BB-1DBOVA1 phages for 1 hour at 37°C. Half of these mixtures was exposed to thermal induction (15 minutes at 42°C). An aliquot (before and after induction) was spread on LB agar plates containing 25 µg/ml chloramphenicol and 100 µg/ml ampicilline and 2% glucose. The numbers of transformants were counted and are listed in table 9.

type of phages	*E.coli* strain	temperature	Number of transformants
pK7C3-1DBOVA1	D1210	37°C	8000
		37°C-42°C	7800
	D1210HP	37°C	8100
		37°C-42°C	8300
pK7C3BB-1DBOVA1	D1210	37°C	7800
		37°C-42°C	7900
	D1210HP	37°C	7600
		37°C-42°C	2

Table 9: Number of transformants when D1210 or D1210HP cells containing ptrc-OprI-OVA were incubated with pK7C3-1DBOVA1 or pK7C3BB-1DBOVA1 phages with or without thermal incubation.

D1210HP cells displaying ovalbumin on their surface are killed by pseudovirions containing phagemid pK7C3BB-1DBOVA1 after thermoinduction. This experiment clearly demonstrates that *coli* cells, expressing an artificial receptor can be recognised and killed by a bacteriophage with an artificial ligand that recognises the artificial receptor.

Example IX: Library versus library screening ("picup" screening)

Fission yeast (*Schizosaccharomyces pombe* p2, h^+, arg^{3-}, ura^{4-}) was grown in YES medium (0.5% (w/v) yeast extract, 3.0% (w/v) glucose + 225 mg/l adenine, histidine, leucine, uracil and lysine hydrochloride). Cells were harvested by low speed centrifugation. 15 g wet cells were washed with 100 ml S-buffer (1.4 M sorbitol, 40 mM HEPES, 0.5 mM $MgCl_2$ adjusted to pH 6.5). After centrifugation

the pellet was resuspended in 100 ml S-buffer containing 10 mM 2-mercaptoethanol and 1 mM PMSF and incubated at 30°C for 10 minutes. After centrifugation, the pellet was resuspended in 60 ml S-buffer containing 1 mM PMSF, and 460 mg Zymolase 20T (ICN Biomedicals) was added to prepare spheroplasts. After incubation for 3 hours at 30°C, the pellet was washed five times with 100 ml S-buffer containing 1 mM PMSF. Spheroplasts were resuspended in 60 ml Tris (25 mM, pH= 7.5), 100 mM NaCl, 2 mM EDTA supplemented with 1 tablet protease inhibitor mix (Boehringer) and lysed by two passages in French press. The supernatant was recovered after centrifugation for 30 minutes at 15.000 rpm in SS34 rotor. 15.5 g solid ammoniumsulfate was added to 30 ml of the supernatant. After incubation on ice for 1 hour, precipitated material was recovered by centrifugation and resuspended in 20 ml PBS. The solution was equilibrated in PBS by passage over PD10 columns. Following this treatment, the protein concentration was determined with Bio-Rad protein assay kit, using BSA as reference protein. Six aliquots, each 5 ml in volume, with a protein concentration of 8 mg/ml, were prepared for camel immunisation.

<u>The immunisation and blood withdrawal scheme is as follows:</u>

Day 0	Collect preimmune serum
Day 0	inject subcutaneously 1 tube (40 mg protein) + complete freund adjuvant
Day 7	inject subcutaneously 1 tube + incomplete freund adjuvant
Day 14	inject subcutaneously 1 tube + incomplete freund adjuvant
Day 21	Collect anticoagulated blood and serum
Day 21	inject subcutaneously 1 tube + incomplete freund adjuvant
Day 28	inject subcutaneously 1 tube + incomplete freund adjuvant
Day 35	inject subcutaneously 1 tube + incomplete freund adjuvant
Day 38	Collect anticoagulated blood and serum

A cDNA library of *Schizosaccharomyces pombe* is constructed by recloning the *S.pombe* cDNA bank that is used in Two-hybrid system (Clontech). The cDNA inserts are amplified with specific primers harbouring restriction enzyme sites compatible for cloning into the multiple cloning site of ptrc-OprI. The library is transformed in UT5600 or in D1210HP electro-competent cells.

The serum immunoglobulins from the immunised animal (day 21 or 38) are passed over protein A and protein G columns to purify the conventional antibodies and the heavy chain antibodies. Each fraction is used in a Western blot to evaluate the presence and titre of anti-*S.pombe* protein immunoglobulins.

Peripheral blood lymphocytes from the immunised camel are prepared using Unisep (WAK Chemie, Germany) from the anticoagulated blood isolated at days 21and 42. The camel heavy chain antibodies (VHH's) from 10^7 lymphocytes are ligated after RT-PCR amplification in the SfiI-NotI sites of the pK7C3 or pK7C3-BB (pK7C3 with the barnase-barstar inversion system) vector and transformed in TG1 as described above, in order to obtain a library of 10^8 individual clones. The VHH phages is prepared by infection of the *E.coli* culture with M13K07 and enriched for virions with a VHH-gpIII fusion by IMAC chromatography (see before).

For the PICUP experiment, 10^7-10^8 UT5600 cells from the cDNA library of *S.pombe* are mixed with 10^{12} phages obtained from the camel VHH library. The mixture is incubated for 1 hour at 37°C and plated on LB agar plates containing 100 µg/ml ampicillin, 25 µg/ml chloramphenicol and 2% glucose. Colonies can only grow on this medium if the UT5600 cells are expressing a *S.pombe* antigen that is recognised and subsequently infected by a virion carrying an antigen-specific VHH. For each colony the VHH insert is sequenced with a primer annealing in the gene pIII sequence, while the cDNA coding for the antigen is directly sequenced with an OprI specific primer. The latter sequence is screened in a BLAST on the *S.pombe* genome sequence database to identify the gene. The specificity of the VHH (having a his-tag) is also tested in a Western blot in which

the *S.pombe* extracted proteins are separated on SDS gels. The presence of the VHH is revealed with an anti-His monoclonal antibody (SEROTEC).

As a positive control, individual colonies from the cDNA library are used in a separate PICUP experiment. One single clone that is capable to produce a fusion protein with the lipoprotein (as seen by Western blot) is grown and challenged with the VHH phages from the library. The VHH from clones growing on Ampicilline and Chloramphenicol are induced with IPTG, extracted from the periplasm and it's binding to the yeast protein tested in Western blot and ELISA.

To eliminate particular antigens dominantly present in the matched pairs (*S.pombe* antigen – specific VHH) or particular VHH over-represented in the matched pairs, the killer phage strategy is used. To this end the UT5600 cells carrying the *S.pombe* cDNA library are incubated with the phages from the pK7C3-BB library of VHH. After infection the suspension is incubated at 42°C to eliminate the *E.coli* cells that are infected with phages. The surviving cells are carrying S.pombe antigens that are less frequent represented in the ptrc-Oprl or pK7C3-BB libraries. In a second step these surviving cells are used to inoculate fresh medium and to restart the PICUP experiment as before.

Example X: Characterization of ovalbumin binders obtained by biopanning and by PICUP.

Binders from experiment VII where $7x10^8$ pK7C3-VHHB phages were mixed with $3x10^7$ UT5600{ptrc-Oprl-OVA} cells (table7a) were characterized into further detail. 18 out of 24 sequences were of 1DBOVA1. This is the only binder that could be obtained after a normal biopanning experiment (all the sequences from 31 clones that were analyzed after the second and the third round of biopanning were from 1DBOVA1). Affinities for ovalbumin were determined in a competitive ELISA (table 10) and range between 4 and 200nM. The affinity of 1DBOVA1 for ovalbumin is 26nM.

Table 10	PANNING	PICUP	K_D (nM)
1DBOVA1	31	18	26
B69	0	1	40
B161	0	1	4
B167	0	1	5
B190	0	1	70
B216	0	1	200
B300	0	1	21

Table 10: Different binders obtained by biopanning and by PICUP. The numbers indicate the frequency of occurrence of the different binders. The affinities were determined in competitive ELISA.

For biopanning, different rounds of selection are performed, and it is documented that the population of phages is biased for clones that grow faster than others (Beekwilder et al, 1999, Gene, 228, 23-31 and de Bruin et al, 1999, Nature Biotechnology, 17: 397-399).

It seems that the PICUP strategy is far more powerful compared to the conventional biopannings in retrieving a larger variety of binders. The sequences of the ovalbumin binders are shown in figure 6.

Example XI: mechanism of pick-up

Cloning of the 1DBOVA1 VHH in the Fdtetdog vector

The 1DBOVA1 VHH (isolated by the biopanning experiment) was amplified with the following primers: GAT GTG CAG CTG CAG GCG TCT GGT GGA GG and GTG TGC GGC CGC TGA GGA GAC GGT GAC CTG. Fragments were digested with PstI-NotI, cloned in the Fdtetdog vector(Zacher, A.N., Stock, C.A., Golden, J.W. and Smith, G.P. (1980) gene 9, 127-140) and transformed in TG1 cells.

Construction of phages with D1 or D2 deleted

GpIII of the phage is a three-domain protein with each domain separated by glycine-rich tetra and pentapeptide repeats and followed by a C-terminal membrane anchor (figure7). Domain 2 (D2) binds to the F-pilus in the F^+ cell. The pilus retracts and domain 1 (D1) binds to the TOL receptors present in the *E.coli* membrane. D1 and D2 were deleted in Fdtetdog by standard PCR-cloning techniques.

Deletion of D1

The VHH part (1DBOVA1) in the Fdtetdog vector was amplified with the following primers: GAT GTG CAG CTG CAG GCG TCT GGT GGA GG and CTG AAT GGG GCC GCG TCG ACT GTT GAA AG. Domain 2 up to the BamHI site in domain 2 was amplified with following primers: CTG AGG GTG GGT CGA CTA AAC CTC CTG AG and CGA ATG GAT CCT CAT TAA AGC. Both PCR products were digested with SalI, ligated, amplified by PCR with the outer primers and cloned in the PstI-BamHI site of Fdtetdog.

Deletion of D2

A fragment was amplified with and without the VHH part (1DBOVA1) up to the beginning of the D2 domain with the following primers: GAT GTG CAG CTG CAG GCG TCT GGT GGA GG and AAT AGG ATC CCC ACC CTC ATT TTC AGG GAT AGC AAG. The PCR product was cloned in the PstI-BamHI site of Fdtetdog.

The resulting constructs are shown in figures 8 and 9.

Construction of phages displaying 1DBOVA1

1DBOVA1 from the Fdtetdog constructs was expressed on M13 in 300 ml 2xTY

medium containing 15 µg/ml tetracycline after overnight culture at 37°C and at 250rpm. Phages were precipitated twice with PEG as described above. The pellet was dissolved in 1 ml PBS containing 0.1% casein and the phages were filtered (0.22µm) before use.

Titer determination

The titer of Fdtetdog phages was determined by incubation of 150µl TG1 cells at OD600nm= 0.5 with 10µl of phages of different dilutions for 30 minutes at 37°C. This was plated on LB agar plates containing 15µg/ml tetracycline. OD260= 1 = 3 x 10^{11} phages/ml.

Display of OprI and OprI fusion's on E.coli

The gene coding for OprI was amplified from the ptrc-OprI plasmid with the following primers: CAG ACC ATG GCC AAC GTT CTG AAA TTC and ATC CGT CTA GAG CTC CAA GCT TGC CTT GCG GCT GGC TTT TTC CAG CAT GCG. The fragment was digested with NcoI-XbaI and cloned in the pBAD/myc-HisB vector of Invitrogen. The ampicillin gene was replaced by a streptomycin-spectinomycin cassette. Therefore the pBAD-OprI vector was amplified with the following primers: CGT TCA CCG ACA AAC and TTC GTT CCA CTG AGC GTC. The streptomycin-spectinomycin cassette was amplified with the following primers: GTG GAA TTC GCC GGC CAA GCG GCG TCG GCT TGA and CCG GAT CCG TGC ACA GCA CTT GTT GTA GAA and cloned by blunt-end ligation in the vector. The resulting plasmid is called pBAD-OprI (figure 10).
The full-length gene encoding hen-egg ovalbumin was amplified with the following primers: GGC AAG CTT GGG CTC CAT CGG TGC AGC AAG C and GGC TCT AGA GGG AAA CAC ATC TGC C. The PCR product was digested with HindIII-XbaI and cloned in the pBAD-OprI vector (pBAD-OprI-OVA).

pBAD-TOLA was constructed by amplifying the TolA gene with the following primers: AGA GAG CCA TGG CAA AGG CAA CCG AAC AAA ACG ACA AGC TC and CGG AAT TCG AAC CTC CTG CCT CTG CGG CCG CTT TTG CTG C and cloning the resulting fragment in the NcoI-EcoRI site of the pBAD-OprI derivative that still contains the ampicilin resistance. Ovalbumin was cloned in this vector as full-length clone or a partial clone, starting at position 424 (aa 142). The resulting plasmids were called pBAD-TOLA-OVA and pBAD-TOLA-partOVA, respectively.

Determination of D1 and D2 dependency of the entry of phages

The pBAD-OprI and pBAD-OprI-OVA plasmids were transformed in DH5α and cultures were started at 37°C in LB medium containing 20 µg/ml streptomycin and 50 µg/ml spectinomycin until the OD600nm= 0.4. Cells were induced with 0.02% arabinose for 30 minutes at 37°C and washed 3x with LB medium as described above. 150 µl cells were mixed with 60 µl Fdtetdog, Fdtetdog-1DBOVA1, Fdtetdog-D1-1DBOVA1, Fdtetdog-D2 or Fdtetdog-D2-1DBOVA1 phages in PBS with an OD260= 1 and incubated at 37°C for 1 hour. The mixture was washed 5x with LB and plated on LB agar plates containing 20 µg/ml streptomycin, 50 µg/ml spectinomycin, 15 µg/ml tetracycline and 2% glucose. The number of colonies are summarized in table 11.

The results clearly indicate that the D1 domain is still needed for entering. D1 binds to the TOL receptors in the *E.coli* membrane. Deletion of the D2 domain results in more colonies. D2 has some affinity for D1 and therefore competes for binding of D1 to the TOL receptors.

Table 11	Fdtetdog	Fdtetdog-1DBOVA1
PBAD-OprI	12	123
PBAD-OprI-ova	9	307

	Fdtetdog-

	D1-1DBOVA1
PBAD-OprI	0
PBAD-OprI-ova	0

	Fdtetdog-D2	Fdtetdog-D2-1DBOVA1
PBAD-OprI	630	567
PBAD-OprI-ova	154	1066

Table 11: number of colonies when 150 µl cells expressing OprI or OprI-ova are mixed with Fdtetdog, Fdtetdog-1DBOVA1, Fdtetdog-D1-1DBOVA1, Fdtetdog-D2 or Fdtetdog-D2-1DBOVA1 phages of OD260= 1.

Example XII: Conjugation induced infection of recombinant *E coli* cells bearing an artificial receptor

Preparation of the immobilized XL1blueMRF'kan cells: Feeder plates were made as follows: A culture of 50 ml XL1blueMRF'kan (Stratagene) cells was grown at 37°C in LB medium containing 25 µg/ml kanamycin until OD600nm= 0.5. Cells were pelleted, dissolved in 1 ml LB medium and mixed with 40 ml top-agarose (0.8%) at 52°C. This mixture was poured on a layer of LB agar. Cells were grown for another 2 hours at 37°C before use.

Construction of pBAD-OprI-TEM: The full-length gene encoding TEM1 (class A β-lactamase from *E.coli*) was amplified with the following primers: CGG AAT TCA AGC TTT GCT CAC CCA GAA ACG CTG GTG and CTG CTC TAG ACA GTT ACC AAT GCT TAA TCA GTG AG. The PCR product was digested with HindIII-XbaI and cloned in the pBAD-OprI vector.

Expression of artificial receptors on the surface of female E. coli cells. The pBAD-OprI-TEM plasmid was transformed in DH5α–rif (DH5α-rif is an in house selected derivative of DH5α that is resistant to 100 µg/ml rifampicin). From these recombinant cells, a culture was grown at 37°C in LB medium containing 20 µg/ml

streptomycin, 50 µg/ml spectinomycin and 100 µg/ml rifampicin until the OD600nm reached 0.4. Cells were induced with 0.02% arabinose for 1 hour at 37°C and washed 3x with LB medium as described above.

Bio-panning and immobilization: 1.5 x 10^9 recombinant bacteria expressing the artificial receptor β-lactamase (TEM) on the surface were mixed with 1.5 x 10^{10} fdtetdog-1DBOVA1 (a-specific control phages) or fdtetdog-TEM13 phages (phages expressing a TEM binder; the TEM binder was isolated in a similar way as the OVA binder) in a total volume of 5 ml and incubated at room temperature for 1 hour. The mixtures were washed 5x with 5 ml LB. The washed cell pellets were re-suspended in 200µl LB and plated on 0.45 µm nitrocellulose filters.

Conjugation and selection of infected cells. Filters containing the immobilized bacteria and the co-immobilized phages were then turned upside down and incubated on feeder plates for 2 hours at 37°C for conjugation. After conjugation the filters were cut into four equal quadrants. Each quadrant was transferred to another selective medium:

- LB agar plates containing 20 µg/ml streptomycin, 50 µg/ml spectinomycin, 100 µg/ml rifampicin and 2% glucose were used do count the number of DH5α-Rif cells encoding a receptor that survived the procedure.
- LB agar plates containing 20 µg/ml streptomycin, 50 µg/ml spectinomycin, 100 µg/ml rifampicin, kanamycin 25 µg/ml and 2% glucose were used do count the number of DH5α-Rif cells encoding a receptor and received the F factor by conjugation.
- LB agar plates containing 10 µg/ml tetracycline and 2% glucose to count the total number of bacteriophages that were co-immobilized with the bacteria.
- LB agar plates containing 20 µg/ml streptomycin, 50 µg/ml spectinomycin, 10 µg/ml tetracycline, 100 µg/ml rifampicin and 2% glucose are used to count the number of DH5α cells, encoding an artificial receptor and containing the

genome of a recombinant bacteriophage.

Results

Recombinant female *E.coli* cell (DH5α, rif^r) were transformed with a pBAD-0prl-TEM, encoding β-lactamase which is expressed as artificial receptor on the surface of *E.coli*. The plasmid renders the recombinant cells streptomicin and spectinomicin resistant.

Said DH5α-Rif cells encoding β-lactamase as the artificial receptor were mixed with recombinant bacteriophages displaying a VHH that has a high affinity for the receptor (Fdtetdog-TEM). As a negative control, these cells were also mixed with phages that display a VHH specific for ovalbumin (Fdtetdog-1DBOVA1). Both mixtures were washed by several rounds of centrifugal precipitation of the bacteria followed by removal of the supernatant, followed by re-suspension in a medium without bacteriophages. Bacteriophages expressing a ligand that is recognizing an artificial receptor co-precipitate with the bacteria expressing this receptor. Bacteriophages that display a ligand that does not recognize an artificial receptor on the host remain in the supernatant and are discarded during the wash procedure. By this procedure, bacteriophages displaying a ligand remain attached on the surface of those *E.coli* cells that express an artificial receptor that specifically binds the ligand.

After a number of wash cycles, the *E.coli* cell pellet (containing the co-precipitated and attached bacteriophages) are re-suspended in a small volume and plated at appropriate dilutions on a (0.45 µm) nitrocellulose filter (millipore, MF-membrane filters), as described above, to immobilize individual cells on one side of the filter. Bacteriophages (attached on the surface of a particular bacterium) are co-immobilized with the bacterium on a filter by this procedure.

These filters, containing the bacteria and the co-immobilized bacteriophages are then used to overlay a confluent layer of male *E.coli* cells (XL1-blue MRF'kan; immobilized in a petri dish using soft agar) for two hours. The filter is placed as

such that the immobilized female cells (DH5α Rif^r) make direct contact with the male cells (XL1-blue MRF'kan) containing a conjugational F factor that renders the host Kanamycin resistant. During this 2-hours period the F factor is efficiently conjugated from the male cells (XL1-blue MRF'kan) to the female cells (DH5α Rif^r) expressing the artificial receptor, rendering these latter cells susceptible to infection with bacteriophage Fd.

After 2 hours, the filters are lifted from the layer of male cells, turned around, cut in four equal quadrants and put on LB plates containing the following antibiotics: Rifampicin, Streptomicin, Spectinomicin, Tetracycline and Kanamicin. *E.coli* cells that are resistant to all these antibiotics are of strain DH5α (Rif^r), contain the plasmid encoding the artificial receptor (Strep^r, Spec^r), contain the F factor (Kan^r) and contain the genome of a recombinant Fd phage (Tet^r). In this way, DH5α cells (Rif+) can be selected that (1) encode the artificial receptor (Strep^r, Spec^r) and (2) contain the genome of a bacteriophage that displays a ligand that is recognized by the artificial receptor.

After overnight incubation at 37°C, the colonies were counted. The results are summarized in the table below.

Number of colonies when bacteriophages displaying an ovalbumin or TEM specific VHH are mixed with DH5α-Rif cells encoding β-lactamase as an artificial receptor

	Recombinant bacteriophage	
	Fdtetdog-1DBOVA1	**Fdtetdog-TEM**
Strep, Spec, Rif[1]	Confluent[2]	Confluent[2]
Strep, Spec, Rif, Kan	Confluent[2]	Confluent[2]
Tet	500	50.000
Strep, Spec, Rif, tet	60	5.000

1. Antibiotics used to select for particular cells. For more details, see *Conjugation and selection of infected cells*
2. Number of colonies > 10^6.

The observation that cells grow confluently on Strep, Spec, Rif en Kan illustrates that the F factor is efficiently conjugated from the male cells (XL1-blue MRF'kan) to the female cells (DH5α Rif^r) expressing the artificial receptor, rendering these cells susceptible to infection with bacteriophage Fd. More accurate counting on appropriate dilutions has shown that about 66% of the DH5α-Rif cells that survive the mix, wash and immobilization step acquire the F factor by conjugation.

About 50.000 Tem specific phages (Fdtetdog-TEM) were co-immobilized with the DH5α Rif^r cells expressing β-lactamase on its surface. If we compare this to the number of aspecific phages (Fdtetdog-1DBOVA1) it can be concluded that this particular wash procedure causes an 100-fold enrichment for phages that recognize the receptor.

By comparing the numbers of specific (5000) *versus* a-specific (60) phages that enter DH5α, expressing the TEM receptor, we conclude that the current procedure allows us to efficiently recover cells expressing an artificial receptor that contain a bacteriophage displaying a ligand that is specifically recognized by this receptor.

Claims

1. Genetically modified bacteriophage, pseudovirion or phagemid capable of entering a host cell by binding of its artificial ligand to an artificial receptor present on said host cell.

2. Genetically modified bacteriophage, pseudovirion or phagemid according to claim 1, carrying a nucleotide sequence encoding an artificial ligand, in condition enabling expression of said ligand at the surface of the bacteriophage, pseudovirion or phagemid.

3. Genetically modified bacteriophage, pseudovirion or phagemid according to claim 1 or 2, in which the artificial receptor is an endogenous cell wall protein of the host cell.

4. Genetically modified bacteriophage, pseudovirion or phagemid according to claim 1 or 2, in which the artificial receptor is a fusion protein whereby the protein sequence or region of said fusion protein, involved in the binding of the receptor with said bacteriophage, pseudovirion or phagemid is free of peptide sequences having 10 or more contiguous amino acid residues involved in the wild type bacteriophage-host cell interaction.

5. Genetically modified bacteriophage, pseudovirion or phagemid according to claim 1-3, where the binding is mediated by an antigen - antibody reaction.

6. Genetically modified bacteriophage, pseudovirion or phagemid according to claim 1-4, in which the said bacteriophage, pseudovirion or phagemid is lytic.

7. Genetically modified bacteriophage, pseudovirion or phagemid according to claim 6, in which the entering in the host cell of said bacteriophage, pseudovirion or phagemid is inducing expression of barnase in the host cell.

8. Genetically modified bacteriophage, pseudovirion or phagemid according to claim 1-7, where the bacteriophage is M13.

9. Genetically modified bacteriophage, pseudovirion or phagemid according to claim 8, where the artificial ligand is a pIII fusion protein.

10. Genetically modified bacteriophage, pseudovirion or phagemid according to claim 9, in which the phagemid is a pK7C3 derived vector

11. Genetically modified bacteriophage, pseudovirion or phagemid according to claim anyone of claims 1 to 4, in which the fusion protein is an pOrpl or a TolA fusion protein.

12. Genetically modified bacteriophage, pseudovirion or phagemid according to claim 11, in which the host cell is transformed with an ptrc-Oprl derived vector.

13. Genetically modified bacteriophage, pseudovirion or phagemid according to claim 12, in which the phagemid is a pK7C3 derived vector.

14. Genetically modified bacteriophage, pseudovirion or phagemid according to claim 5, in which the antibody is a camelid derived antibody, or is a functional fragment thereof, including a fragment comprising all or part of the VHH chain of a camelid heavy chain antibody.

15. Use of a bacteriophage, pseudovirion or phagemid according to claim 1-14 to detect and/or eliminate a specific bacterial population.

16. Use of a bacteriophage, pseudovirion or phagemid according to claim 1-14 to detect a artificial receptor- artificial ligand interaction.

17. Use of a bacteriophage, pseudovirion or phagemid according to claim 15 to screen an antigen and/or antibody library.

18. Method for selecting artificial receptor - artificial ligand interactions, comprising:

- growing a host cell or a mixture of host cells displaying one or more artificial receptors,
- contacting said host cell or said mixture with a genetically modified bacteriophage, pseudovirion or phagemid or a mixture of genetically modified bacteriophages, pseudovirions or phagemids with one or more artificial ligands,

- selecting those cells that have been entered by one or more bacteriophages, pseudovirion of phagemid.

19. Method for selecting artificial ligand-artificial receptor interactions according to claim 18, in which the host cell is transformed with an ptrc-Oprl derived vector.

20. Method for selecting artificial ligand-artificial receptor interactions according to claim 18 or 19, in which the phagemid is a pK7C3 derived vector.

21. Genetically modified host cell, carrying a nucleotide sequence encoding an artificial receptor in conditions enabling that the artificial receptor be expressed at the surface of the host cell, said host cell being further transformed with a nucleotide sequence encoding said artificial ligand whereby said nucleotide sequence encoding the ligand entered the host cell as a consequence of the interaction between said artificial ligand and a protein sequence or region on said artificial receptor.

22. Genetically modified host cell according to claim 21, wherein the nucleotide sequence encoding the artificial receptor and/or the nucleotide sequence encoding the artificial ligand are not known.

23. Genetically modified host cell according to claim 21 or 22, which is a gram-negative bacterium, especially an E coli cell of the F^- strain.

24. Genetically modified host cell according to any of claims 21 to 23, wherein the nucleotide sequences of the artificial receptor and the nucleotide sequence for the artificial ligand are respectively coding sequences of an antibody or a functional fragment thereof and coding sequence of an antigen, or are respectively coding sequences of an antigen and coding sequence of antibody or a functional fragment thereof.

25. Genetically modified host cell according to claim 24, wherein the functional antibody fragment is a VHH fragment of a camelid antibody or a functional portion of said VHH.

26. Genetically modified host cell according to anyone of claims 21 to 25, wherein the nucleotide sequence encoding the artificial receptor comprises a sequence encoding OprI or TolA or a part of OprI or a part of TolA sufficient to enable the exposure, at the surface of the host cell, of a protein sequence or region capable of interacting with the artificial ligand.

27. A kit comprising a genetically modified host cell according to anyone of claims 21 to 26 bacteriophage, pseudovirion or phagemid according to anyone of claims 1 to 20, or comprising a host cell and/or a bacteriophage, pseudovirion or phagemid and/or a cloning vector enabling the construction of said genetically modified host cell according to anyone of claims 21 to 26.

28. A kit according to claim 27 for in vivo panning of antibody or antibody fragment library, or antigenic sequences library.

29. A kit according to claim 27 or 28, for the simultaneous in vivo panning of both an antibody fragment library, and an antigenic sequences library.

Figure 1: outline of the screening of an antigen - antibody library

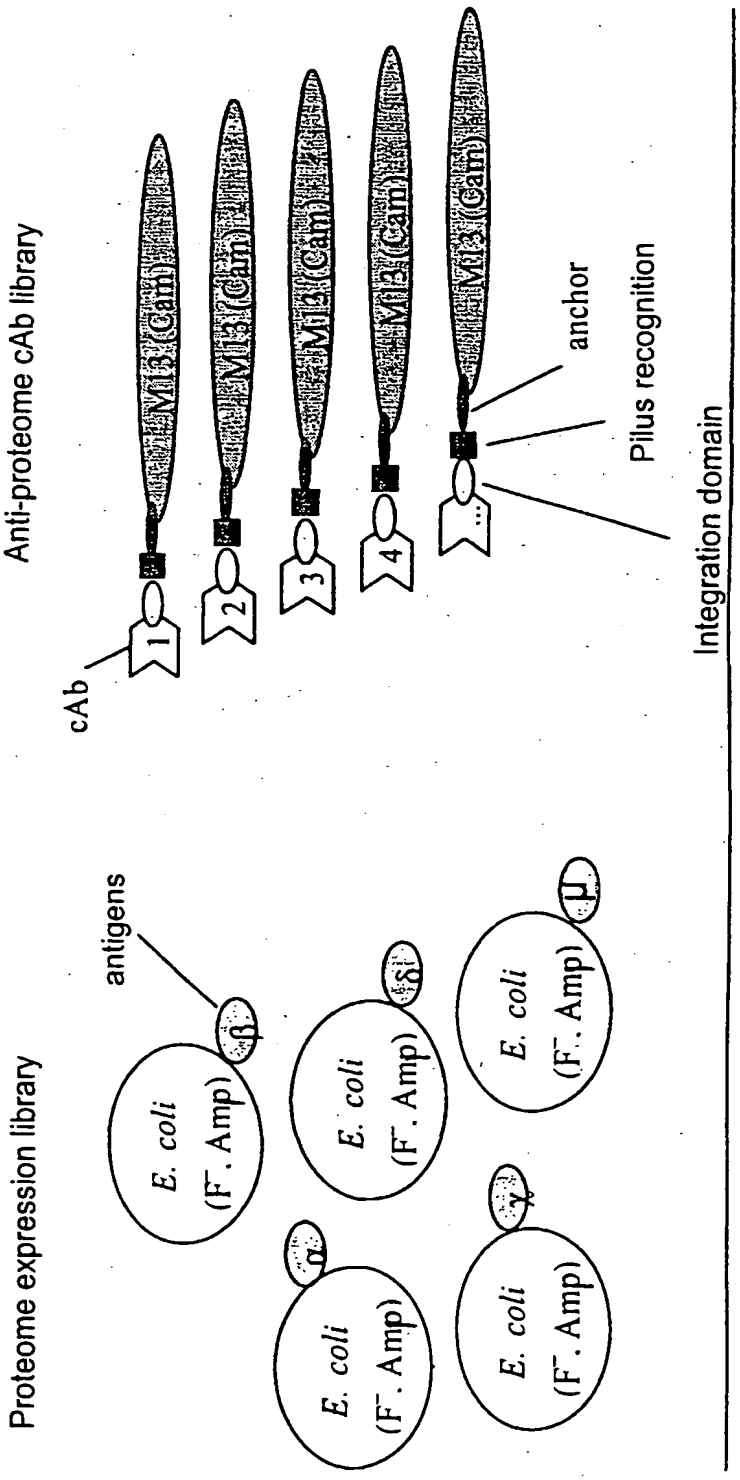


Figure 2

Phagemid : pK7C3

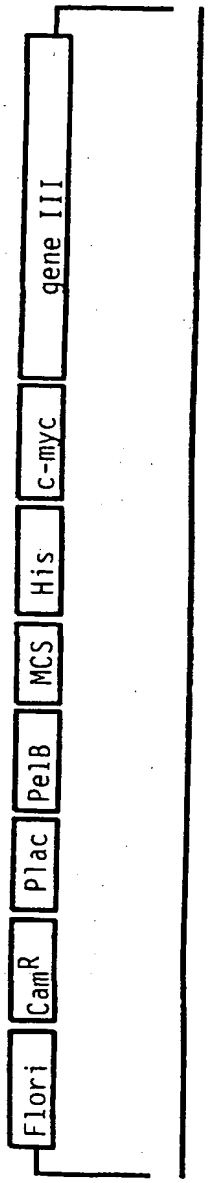


Figure 3

Ptrc-OprI

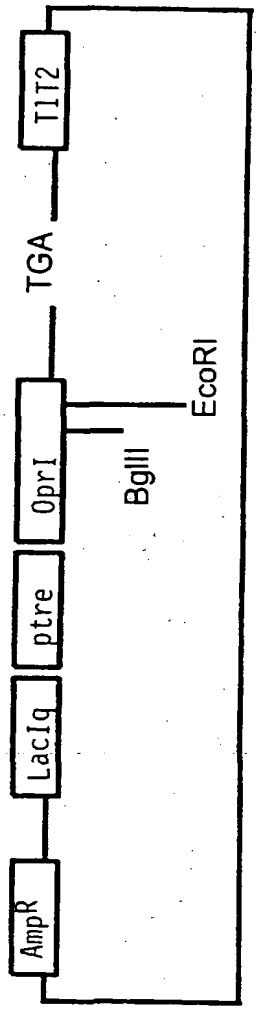
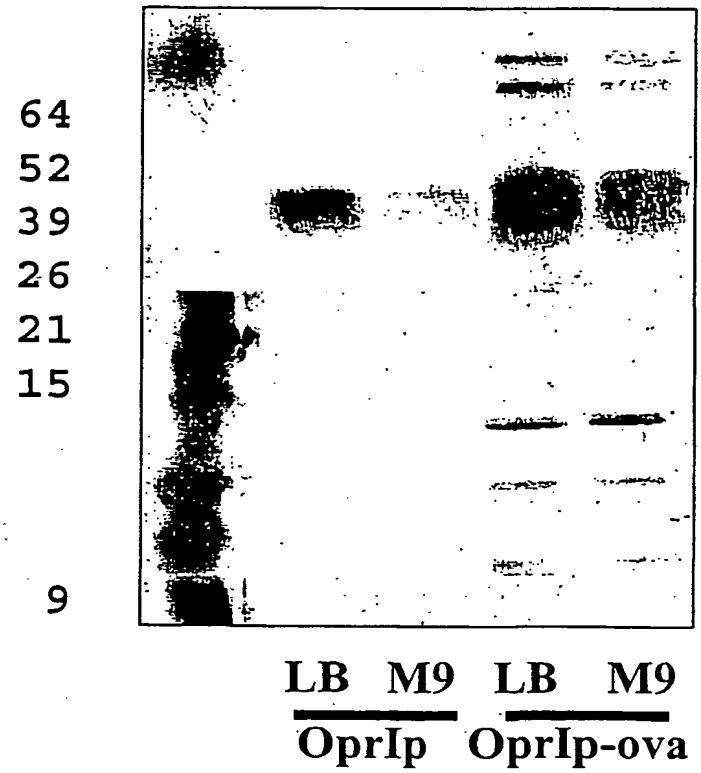


Figure 4: Results Western blot



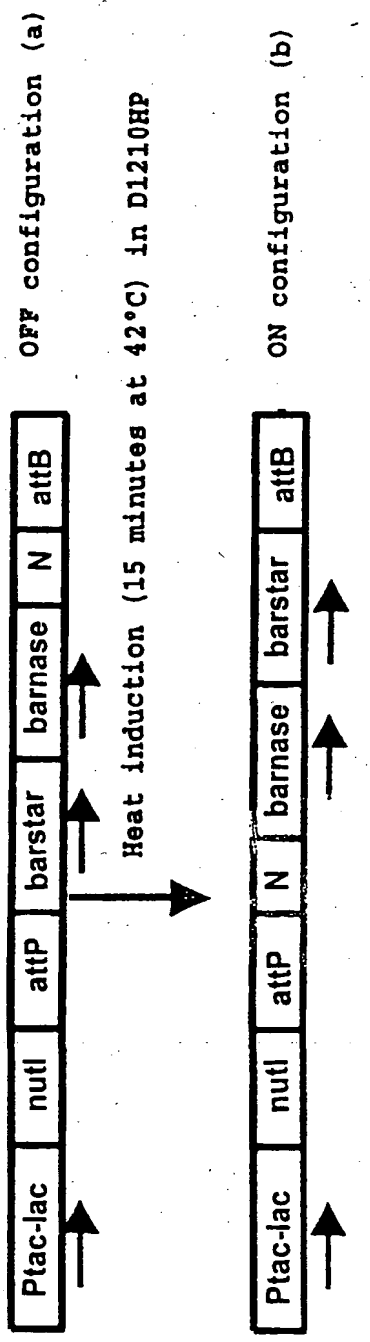


Figure 5a-b: barnase-barstar inversion cassette

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B69       DVQLVESGGGSVQAGGSLRLSCTAP GFTSNSCGMD       WYRQAAGKQREWVS
B161      QVQLVESGXXXVQXXXXXXXXSCXXXXXXXXXXXXXXXXXXXXXXXXXXXPGKEREFVS
B167      GHGAGIGRESRGCAGGSLRLSCIYS GGWYNDLCIG       WFRQAPGKEREGVA
B190      QVQLVESGGGSVQAGGSLRLSCAVS GYTHSNYYMG      WFRQAPGKEREGVA
B216      QVQLVESGGGLVETGGSLKLACVGD FESDASGYSFADSEMG WYRQAPGNECAMVS
B300      DVQLVESGGGSVQAGGSLRLSCTAS GFTFDDFEMG       WYHQAPGNERELVS
          1         11          22                    37     44 47

          <-----CDR2------> <----------framework 3---------->
1DBOVA1   AIWRGGNNPYYADSVKG RFTISQDNAKNIVSLLMNSLKPEDTAIYYCAA
B69       SISTDGS TSYADSVKG RFTISKDKAKDTVYLQMNSLKPEDTAIYYCAA
B161      SVRRYGS TNYADSVKG RFTISQDNAKNTVYLQMNSLKADDTAIYYCKA
B167      AIKRGDGMTYYADSVKS RFTISRVNAENTVYLTLNSLKPDDTAVYYCAA
B190      AIRSGSASAHYADSVKG RFTISQDNAKNTVYLLMTSLKPEDTAVYYCAT
B216      TITVDDRVE YGDSVKG RFTISHDTLTGTVYLDMDNLAPEDTAIYYCTT
B300      TISSDGDTS YTESVKG RFTMSRDNAKKTVYPQMDSLKPEDTGMYYCAA
                                                        92

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B161      VCSVRDRSDRYPF           WGQGTQVTVSS
B167      GDYSCGYCYCKGFDY         WSRGTQVTVSS
B190      RDFSVYFAYDPLDPDAYRF     WGQGTQVTVSS
B216      SYSPYVGCRPEDDYRHLEI     WGRGTQVTVSS
B300      VEDTGSGGLINALGA         WGQGTQVTVSS
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Figure6: Sequences of the ovalbumin binders obtained by biopanning and PICUP of CAMELB library

Figure7 : fdtetdog

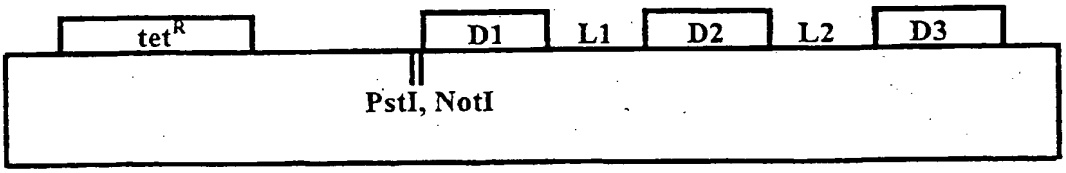


Figure8: Fdtetdog-D1

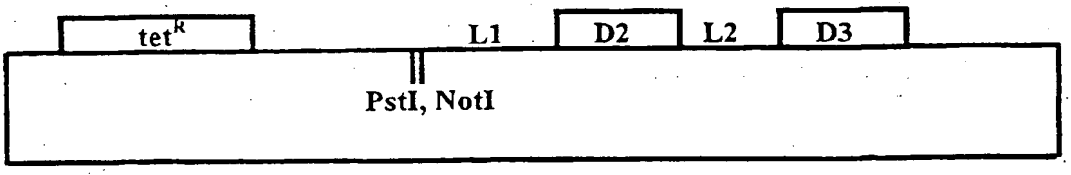


Figure9: Fdtetdog-D2

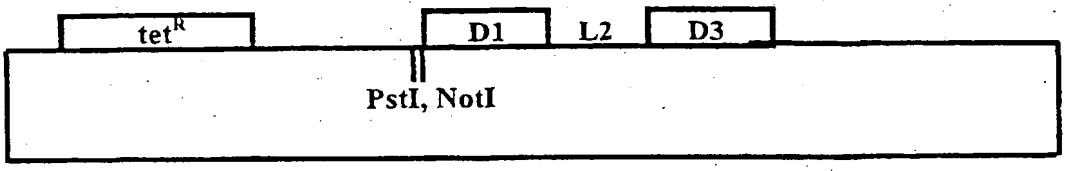


Figure10 : pBAD-OprI

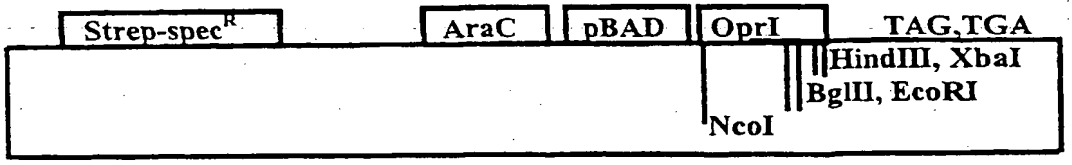
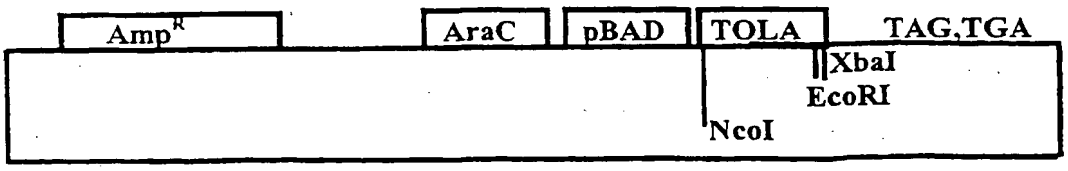


Figure11 : pBAD-TOLA



SEQUENCE LISTING

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<120> RECOMBINANT PHAGES CAPABLE OF ENTERING HOST CELLS VIA SPECIFIC INTERACTION WITH AN ARTIFICIAL RECEPTOR

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35 40 45

Ala Ile Trp Arg Gly Gly Asn Asn Pro Tyr Tyr Ala Asp Ser Val Lys
50 55 60

Gly Arg Phe Thr Ile Ser Gln Asp Asn Ala Lys Asn Ile Val Ser Leu
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Leu Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Ile Tyr Tyr Cys Ala
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<210> 18
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<213> Camelus dromedarius

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			20					25					30		
Gly	Met	Asp	Trp	Tyr	Arg	Gln	Ala	Ala	Gly	Lys	Gln	Arg	Glu	Trp	Val
		35					40					45			
Ser	Ser	Ile	Ser	Thr	Asp	Gly	Ser	Thr	Ser	Tyr	Ala	Asp	Ser	Val	Lys
	50					55					60				
Gly	Arg	Phe	Thr	Ile	Ser	Lys	Asp	Lys	Ala	Lys	Asp	Thr	Val	Tyr	Leu
65					70					75					80
Gln	Met	Asn	Ser	Leu	Lys	Pro	Glu	Asp	Thr	Ala	Ile	Tyr	Tyr	Cys	Ala
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<210> 20
<211> 129
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20 25 30

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
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50 55 60

Thr Asn Tyr Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Gln Asp
65 70 75 80

Asn Ala Lys Asn Thr Val Tyr Leu Gln Met Asn Ser Leu Lys Ala Asp
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Ser

<210> 21
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<213> Camelus dromedarius

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Cys Ile Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly Val
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Ala Ala Ile Lys Arg Gly Asp Gly Met Thr Tyr Tyr Ala Asp Ser Val
50 55 60

Lys Ser Arg Phe Thr Ile Ser Arg Val Asn Ala Glu Asn Thr Val Tyr
65 70 75 80

Leu Thr Leu Asn Ser Leu Lys Pro Asp Asp Thr Ala Val Tyr Tyr Cys
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Tyr Trp Ser Arg Gly Thr Gln Val Thr Val Ser Ser
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<400> 22

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35 40 45

Ala Ala Ile Arg Ser Gly Ser Ala Ser Ala His Tyr Ala Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Gln Asp Asn Ala Lys Asn Thr Val Tyr
65 70 75 80

Leu Leu Met Thr Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Thr Arg Asp Phe Ser Val Tyr Phe Ala Tyr Asp Pro Leu Asp Pro
100 105 110

Asp Ala Tyr Arg Phe Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser
115 120 125

<210> 23
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<400> 23
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20 25 30

Tyr Ser Phe Ala Asp Ser Glu Met Gly Trp Tyr Arg Gln Ala Pro Gly
35 40 45

Asn Glu Cys Ala Met Val Ser Thr Ile Thr Val Asp Asp Arg Val Glu
50 55 60

Tyr Gly Asp Ser Val Lys Gly Arg Phe Thr Ile Ser His Asp Thr Leu
65 70 75 80

Thr Gly Thr Val Tyr Leu Asp Met Asp Asn Leu Ala Pro Glu Asp Thr
85 90 95

Ala Ile Tyr Tyr Cys Thr Thr Ser Tyr Ser Pro Tyr Val Gly Cys Arg
100 105 110

Pro Glu Asp Asp Tyr Arg His Leu Glu Ile Trp Gly Arg Gly Thr Gln
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Val Thr Val Ser Ser
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<210> 24
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<400> 24

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Ser Leu Arg Leu Ser Cys Thr Ala Ser Gly Phe Thr Phe Asp Asp Phe
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Glu Met Gly Trp Tyr His Gln Ala Pro Gly Asn Glu Arg Glu Leu Val
         35                  40                  45

Ser Thr Ile Ser Ser Asp Gly Asp Thr Ser Tyr Thr Glu Ser Val Lys
     50                  55                  60

Gly Arg Phe Thr Met Ser Arg Asp Asn Ala Lys Lys Thr Val Tyr Pro
 65                  70                  75                  80

Gln Met Asp Ser Leu Lys Pro Glu Asp Thr Gly Met Tyr Tyr Cys Ala
                 85                  90                  95

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Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser
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<221> misc_feature
<222> (1)..(35)
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<400> 28
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INTERNATIONAL SEARCH REPORT

Inte ial Application No
PC I/EP 00[redacted]77

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/70 C12N15/62 C07K16/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EMBASE, EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 34006 A (BORDIGNON CLAUDIO ;GENERA S P A (IT)) 8 July 1999 (1999-07-08)	1-6, 16-18, 21,22, 27-29
Y	the whole document	14,23-25
X	WO 90 01870 A (US ARMY) 8 March 1990 (1990-03-08)	1-6, 16-18, 21,22, 27-29
	the whole document	
Y	WO 94 04678 A (CASTERMAN CECILE ;HAMERS RAYMOND (BE)) 3 March 1994 (1994-03-03) the whole document	7,14, 23-25
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
22 January 2001	09/02/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Panzica, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/09277

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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